

# Comparison of Biochemical, Molecular, and Visual Methods to Quantify *Phaeocryptopus gaeumannii* in Douglas-Fir Foliage

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## ABSTRACT

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A recent epidemic of Swiss needle cast along the Oregon coast has prompted efforts to quantify foliar infection and colonization of the causal agent *Phaeocryptopus gaeumannii*. In this paper, we compare four methods to quantify colonization of Douglas-fir foliage by *P. gaeumannii*: fruiting body abundance, ergosterol content, dot blot analy-

sis, and TaqMan based real-time quantitative polymerase chain reaction (PCR). Results from the four techniques were all significantly correlated. Fruiting body density and quantitative PCR are two methods least affected by the presence of other needle fungi and had the highest correlation. The methods also were used to compare foliage colonization in nine field sites exhibiting a range of disease severity. All four methods provided evidence that sites differed in the degree of fungal colonization, but only quantitative PCR consistently separated sites with moderate to severe levels of disease from sites with low disease estimated by foliage color, canopy density, and growth measurements.

Swiss needle cast of Douglas-fir historically has caused economically significant disease only where Douglas-fir has been planted as an exotic species or Christmas tree crop (5,8,14,15). The causal agent of the disease, *Phaeocryptopus gaeumannii* (Rohde) Petrak, is believed to be endemic to the Pacific Northwest and is ubiquitous on Douglas-fir throughout the region. Recently however, Swiss needle cast has been implicated in a serious decline of Douglas-fir along the coastal region of Oregon (11). Severely diseased plantations are chlorotic, lose needles prematurely, and suffer from reduced growth. Our objective, quantitative measurement of both disease symptoms and pathogen abundance, is a challenging aspect of research on this disease, which has been particularly hampered by inadequate methods for measuring the extent of colonization by the pathogen.

Accurate measurement of pathogen abundance is critical to epidemiological studies of plant pathogens. *P. gaeumannii* produces ascomatal fruiting bodies (pseudothecia) in the stomata of infected needles (23). Incidence of needles bearing pseudothecia can be a useful measure of infection and pathogen distribution (13). However, because incidence in many Douglas-fir stands near the Oregon coast is near 100%, this measure may not differentiate small but important differences in disease severity. The proportion of stomata occupied by pseudothecia gives a good measure of colonization (11), but since pseudothecial initials usually do not become visible until approximately 9 months after spring ascospore infection of newly emerging needles (4), pseudothecia cannot be counted reliably until the spring following initial ascospore infection. The need to count both stomata and pseudothecia also imposes serious labor and statistical challenges, because many stomata on many needles must be counted. This becomes particularly troublesome when the incidence of infected needles is low and distribution of fruiting bodies is sporadic.

Therefore alternative methods, ergosterol content and pathogen DNA, were investigated for quantification of colonization.

Ergosterol is a cell membrane sterol found only in higher fungi. Although ergosterol cannot be used to discriminate between different fungal species, it has previously been used as a quantitative measure of both endophytic and pathogenic fungal biomass in forest foliage (18–20).

Species-specific DNA hybridization probes have been designed to quantify colonization of plant pathogenic fungi in host tissue (7,10,16). In contrast to ergosterol measurements, DNA probes can be designed that do not react with organisms other than the target species. Quantification is based on densitometric measurement of labeled probes hybridized to samples and compared with a standard curve of target DNA.

Real-time quantitative polymerase chain reaction (PCR) is the most recent development in quantitative methods. Unlike endpoint quantitative PCR, real-time PCR monitors PCR products as they accumulate in the exponential phase, before reaction components become limiting. The most frequently employed application of this technique utilizes TaqMan (Perkin-Elmer Applied Biosystems, Foster City, CA) chemistry (9,12,17) in conjunction with a Sequence Detection System (Perkin-Elmer Applied Biosystems). The fluorogenic TaqMan probe, labeled on opposite ends with a reporter dye and a quencher, anneals to the complementary sequence of the single-stranded DNA. Extension to the probe from one primer reaches the 5' end of the probe to release the fluorogenic activity. During the extension phase of the PCR, the 5'→3' exonuclease activity of *Taq* DNA polymerase cleaves only annealed probe molecules. Release of the reporter dye results in a fluorescent signal which is measured by the sequence detection system during each cycle of the PCR process. TaqMan chemistry has contributed to the development of extremely specific, sensitive, and accurate assays to quantify pathogen infection in soybean seeds (29), the roots of both crop plants and forest trees (1), and Douglas-fir foliage (28).

The purpose of this study is to evaluate the precision, advantages, and disadvantages of several methods to quantify colonization levels of *P. gaeumannii* in Douglas-fir foliage. We

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compare pseudothecia density, ergosterol content, and two methods of measuring *P. gaemannii* DNA in Douglas-fir needles and relate these to disease symptoms observed in nine field sites located within the center of the Swiss needle cast epidemic in Oregon.

## MATERIALS AND METHODS

**Field sites, disease impacts, and sample collection.** Nine disease-monitoring sites were established in Tillamook County, Oregon in 1996 (11). Characterization of relative disease severity in these sites is based on Hansen et al. (11). Although the pathogen was present in sites classified as healthy, disease symptoms were minimal. The sites were grouped in three clusters by latitude (Table 1). The three sites in each cluster were located in Douglas-fir plantations exhibiting a range of symptom severity. Plantations consisting of trees of the same age and seed source were selected where possible. Sites in the South Cluster were all U.S. Department of Agriculture-Forest Service progeny test plantations; 10 trees of each of two families were randomly selected for measurements in each plantation. Sites of the Tillamook Cluster and the North Fork site of the North Cluster were planted with seedlings from the same bulk seed lot from the "Boundary" seed collection area of the Coast Range, at approximately 600-m elevation. Ten trees were randomly selected in each of the Boundary seed source plantations. The North Cluster also included two sites located in the Oregon Department of Forestry progeny test plantations. Ten trees of each of two families (different from the South Cluster sites) were selected in each of the latter plantations.

Each selected tree was monitored for growth, symptom, and pseudothecium development between 1998 and 1999. Tree volume was calculated from diameter and height measured in 1998 with a formula derived for young-growth trees (3). Crown density and chlorotic discoloration were measured by two observers just before bud break in spring 1999 and the average was recorded. Crown density, an estimate of needle retention, was compared with standardized diagrams and estimated in 5% increments as the percentage of sunlight being blocked by the live crown. Discoloration, an estimate of chlorosis, was described from foliage in the fifth whorl from the top of the tree in 4 classes: 1 = normal green, 2 = slight yellowing, 3 = moderate yellowing, and 4 = extremely yellow or yellow-brown. For quantitative assays, secondary and tertiary branches from the fifth whorl from the apex of each tree were collected just before bud break in spring 1999. One-year-old needles were stripped from the branches, mixed, and stored at  $-20^{\circ}\text{C}$ .

**Pseudothecia density.** Ten 1-year-old needles bearing pseudothecia were randomly selected from each tree. Needles were affixed to index cards with double-sided adhesive tape for microscope examination. Pseudothecia were counted for three regions (basal, medial, and apical) for each needle. From each region, a single row of 80 stomata was randomly chosen for pseudothecium counting. The 30 regions from each tree sample were averaged, and the data were presented as the proportion of stomata occupied by pseudothecia.

**Ergosterol extraction and quantification.** Ergosterol was extracted as previously described (19) from  $\approx 125$  mg of frozen foliage and quantified by high-performance liquid chromatography. All ergosterol contents are reported on a per unit dry weight basis. From each analyzed sample, a subsample was used to create a fresh-to-dry weight ratio for determination of ergosterol sample dry weights.

**DNA extraction.** Ten needles selected randomly from each tree were placed into a 2-ml microcentrifuge tube with two 5-mm glass beads frozen in liquid nitrogen and pulverized in a Mini-Bead-beater (Biospec Products, Bartlesville, OK) for 30 s at 4,200 rpm. After pulverization, samples were incubated in 1.5 ml of extraction buffer (2% cetyltrimethylammonium bromide [CTAB], 100 mM Tris, pH 8.0, 20 mM  $\text{Na}_2\text{EDTA}$ , pH 8.0, 1.4 M NaCl, 1% polyvinylpyrrolidone, and 0.1% 2-mercaptoethanol) at  $65^{\circ}\text{C}$  for 2 h. The DNA was purified in 24:1 chloroform/isoamyl alcohol, precipitated from the aqueous phase by the addition of isopropanol, washed in 70% ethanol, and resuspended in 1 ml of TE (5 mM Tris, pH 8.0, and 0.5 mM  $\text{Na}_2\text{EDTA}$ ).

**Development of DNA probe for dot blot analysis.** Probes were developed from *P. gaemannii* random amplified polymorphic DNA (RAPD) bands, a technique that amplifies arbitrary sequences throughout the genome (26). Candidate *P. gaemannii*-specific bands were identified by comparing amplification products of *P. gaemannii* with several other common Douglas-fir foliar fungi. Total genomic DNA was extracted from pure cultures of *P. gaemannii* and several of the most common epiphytic and internal fungi isolated from Douglas-fir needles (22) (Fig. 1). Fungal cultures were maintained on potato dextrose agar (Difco Laboratories, Detroit). Each sample was amplified in a 20- $\mu\text{l}$  volume containing 1 unit of amplitherm DNA polymerase (Epicentre Technologies, Madison, WI), 1 $\times$  amplitherm buffer, 2.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  each dNTP, 750 nM RAPD primer, and 2 $\times$  enhancer buffer (Epicentre Technologies), and up to 300 ng of genomic DNA. Six RAPD primers were tested and subsequently used for developing hybridization probes 709, 743, 846, 857, 886 (University of British Columbia, Vancouver), and B02 (Qiagen

TABLE 1. Estimated means of Swiss needle cast symptoms and *Phaeocryptopus gaemannii* colonization measurements at each Swiss needle cast field site in Douglas-fir plantations in the vicinity of Tillamook, OR

Site	Visual disease severity <sup>x</sup>	Crown density (%) <sup>y</sup>	Discoloration score <sup>y</sup>	Volume ( $\text{m}^3$ ) <sup>y</sup>	Pseudothecia density (%) <sup>z</sup>	TAQMAN2 (pg/ng) <sup>z</sup>	Ergosterol (mg/g) <sup>y</sup>	DNA probe (ng) <sup>z</sup>
North Cluster								
Acey Creek progeny	Healthy	46.0 a	2.1 a	0.08 a	2.6 a	0.89 a	8.35 a	41.46 a
Coal Creek progeny	Moderate	41.5 a	2.3 a	0.06 ab	5.7 b	1.27 a	8.18 a	50.64 a
North Fork	Severe	36.7 a	3.0 b	0.05 b	13.1 c	3.75 b	8.90 a	88.38 b
Tillamook Cluster								
Upper Stone	Healthy	44.5 a	1.7 a	0.13 a	4.1 a	1.65 a	5.38 a	66.87 a
Lower Stone	Mild	36.7 a	1.9 a	0.13 a	5.1 a	1.80 a	9.37 b	57.92 a
Juno Hill	Severe	17.0 b	4.0 b	0.02 b	21.2 b	10.12 b	21.42 c	284.39 b
South Cluster								
Limestone progeny	Healthy	51.3 a	1.5 a	0.05 a	2.7 a	0.50 a	4.65 a	23.66 a
Cedar progeny	Mild	43.3 ab	1.9 b	0.05 a	1.6 b	0.46 a	6.11 b	15.59 a
Salal progeny	Moderate	42.1 b	2.2 b	0.03 b	2.5 ab	0.78 b	6.13 b	45.99 b

<sup>x</sup> Visual disease severity was estimated in 1996 (11) by overall impressions of needle retention, chlorosis, and growth.

<sup>y</sup> Comparisons between sites were performed with Fisher's 95% LSD. Sites within clusters that were significantly different ( $P < 0.05$ ) are distinguished by different letters.

<sup>z</sup> Comparisons between sites were performed with Welch's *t*-tests. Sites within clusters that were significantly different ( $P < 0.05$ ) are distinguished by different letters.

Operon, Alameda, CA). Reaction conditions were 40 cycles of 45 s at 92°C denaturing, 45 s at 36°C annealing, and 60 s at 72°C extension. The reaction products were examined side-by-side on 2% (wt/vol) TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) agarose gels to identify bands that were specific for *P. gaeumannii*. One candidate amplicon from each RAPD primer was excised from the gel and purified from the agarose matrix (QIAquick gel extraction kit; Qiagen Operon). The purified PCR products were labeled nonradioactively with alkaline phosphatase according to the manufacturer's directions (AlkPhos Direct Labeling System; Amersham Biosciences, Piscataway, NJ).

For dot blots, DNA samples were denatured in 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 200 mM NaOH and applied with mild suction to Hybond-N+ nylon membranes (Amersham Biosciences). After rinsing the wells with 1× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaPO<sub>4</sub>, and 1 mM EDTA [pH 7.7]), the membranes were air dried, UV cross-linked at 12,000 µJ (UV Stratalinker 1800; Stratagene, La Jolla, CA), and hybridized overnight to the labeled PCR products at 60°C. After hybridization, membranes were washed twice at 55°C and twice at room temperature according to the manufacturer's instructions for the AlkPhos labeling kit. Chemiluminescent signal was generated with CDP-Star (Amersham Biosciences). Membranes were exposed for 1 h to Kodak Biomax ML film (Kodak, Rochester, NY).

For each of the six candidate probes, specificity was characterized on two replicate dot blots prepared from 1-µg total genomic DNA extracts of each of the fungal species used to identify candidate probes as well as total genomic DNA of uninfected, newly flushed Douglas-fir needles. To ensure that candidate probes could detect *P. gaeumannii* in infected foliage, another dot blot was prepared in which dots had increasing amounts of *P. gaeumannii* DNA as well as dots with constant amounts (10 µg) of the total DNA extracted from infected Douglas-fir needles (data not shown).

**Dot blot hybridization assay.** Assay samples consisted of 10 randomly chosen 1-year-old needles from each site tree. DNA extractions were performed as described previously, except 10 µl of each sample was retained for additional quantitative PCR analysis. Dot blotting, probe hybridization, and signal generation were performed as described previously, except approximately 10 µg of each sample was applied to the membrane. In addition, a blot of duplicate, known amount of *P. gaeumannii* was included in each hybridization to serve as a quantification standard. Signal intensities were determined by scanning films with a Molecular Dynamics Personal Densitometer model PDSI and analyzing the data with ImageQuaNT 5.0 (Amersham Biosciences). Absolute amounts of *P. gaeumannii* DNA were estimated from signal intensities by interpolation to internal standard curves.

**TaqMan real time quantitative PCR development.** TaqMan probe/primer sets were designed as previously described (27,28). Briefly, the *P. gaeumannii* probe/primer set was based upon sequence differences between the low copy number β-tubulin gene of six isolates of *P. gaeumannii* versus two to three isolates each of the Douglas-fir needle fungi listed in Figure 1. The specificity and sensitivity of the *P. gaeumannii* probe/primer set was tested on genomic DNA extracted from the isolates described previously, as well as uninfected Douglas-fir needles and infected needles collected both before and after pseudothecial development. The Douglas-fir probe/primer set was based upon a LEAFY/FLORICAULA-like gene involved in floral development (2,25) and served as an endogenous control.

**TaqMan assay PCR conditions and analysis.** Reaction volumes were 25 µl (5 µl of DNA template, 1× TaqMan Universal Master Mix [Applied Biosystems], 50 nM each TaqMan probe, and 60 nM of each primer) performed in MicroAmp optical 96-well plates with caps (Applied Biosystems). Two controls lacking DNA were included in each assay to confirm that chemical stock solutions were not contaminated with template DNA. Real-time

quantitative PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) programmed according to the universal thermal cycler protocol (2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 s and 1 min at 60°C).

Assay standards were obtained from uninfected, greenhouse-grown Douglas-fir needles and *P. gaeumannii* grown in pure culture. DNA was extracted as described previously except ribonuclease A treatment was included. After quantitation by OD<sub>260</sub>, both host and fungal DNA samples were mixed and 10-fold serial dilutions were prepared to serve as one-tube quantification standards for each probe/primer set to be included in each assay. *P. gaeumannii* standards ranged from 1 pg to 10 ng, and Douglas-fir standards ranged from 100 pg to 1,000 ng. The standards were run in duplicate for each assay and used to calculate regression equations to quantitate unknown samples by interpolation. *P. gaeumannii* quantities were normalized to the amount of Douglas-fir host DNA present in individual samples to compensate for pipetting errors and both DNA extraction and PCR amplification efficiencies.

To determine whether *P. gaeumannii* could be detected before appearance of fruiting bodies, samples of current year needles were collected between July 1999 and February 2000 at the Juno Hill and Upper Stone sites, the two sites exhibiting high and low symptom extremes, respectively, within the three Tillamook Cluster sites (Table 1). Needles were stored at -20°C, and DNA was extracted from a subsample of 10 needles as described previously.

Comparisons of average colonization among sites were made from two separate DNA extractions from study site samples. The first set (TAQMAN1) consisted of the same set of randomly chosen needles used for the DNA probe hybridization assay (needles selected without regard to visible signs of the pathogen). The second set (TAQMAN2) was randomly selected from a pool of pseudothecia-bearing needles for which visual pseudothecia density estimates were made prior to DNA extraction. DNA from both sets was extracted as described previously, except needles used for TAQMAN2 were removed from the freezer at irregular intervals to count pseudothecia and were finally air dried for approximately 3 weeks prior to extraction.

**Statistical analyses.** Data were analyzed with the statistical package Statgraphics Plus 4.0 (Statistical Graphics Corp., Rockville, MD). All comparisons except discoloration scores were made after log transformation of the data. Sites were compared by analysis of variance using Fisher's protected least significant differences (LSD) for measurements of ergosterol, crown density, discoloration, and wood volume. Because variances between some sites were unequal, comparisons of pseudothecia density, quan-



**Fig. 1.** Replicate dot blots demonstrating the specificity of the DNA probe. Each circle contains 1 µg of total genomic DNA. The darker the dot the more probe hybridized to the target DNA. The probe was tested against extracts of pure cultures of *Phaeocryptopus gaeumannii*, seven fungi commonly isolated from Douglas-fir foliage, and Douglas-fir needles.

titative PCR, and the DNA probe between sites were made with Welch's *t*-tools. Correlations between disease impact measurements and quantitative methods were tested on individual tree data using Pearson correlation with Dunn-Sidak probabilities.

## RESULTS

**Disease impacts.** There were measurable differences in disease impacts between sites (Table 1). In each site cluster, trees at sites characterized as severely diseased were more chlorotic, had thinner crowns, and reduced growth when compared with those from sites characterized as healthy ( $P < 0.05$ ). For example, the average crown density at North Fork was approximately 20% less than at Acey Creek (95% confidence interval from 2 to 36%) in the North Cluster. Average crown density at Juno Hill, the site with the most severely diseased trees by all measures, was 63% less than at Upper Stone (52 to 71%) in the Tillamook Cluster, and tree crowns at Salal were 21% (6 to 34%) less dense than at Limestone in the South Cluster. Average wood volumes at North Fork, Juno Hill, and Salal were approximately 50% (22 to 68%), 87% (78 to 92%), and 57% (40 to 70%) less than corresponding healthy sites. Across all sites, increased chlorosis was significantly correlated ( $P < 0.0001$ ) with reductions in both canopy density ( $R = -0.70$ ) and wood volume ( $R = -0.47$ ), and canopy density was significantly correlated with wood volume ( $R = 0.56$ ;  $P < 0.0001$ ).

**Pseudothecia density.** North Fork and Juno Hill, the two sites with severe symptoms, both differed significantly from sites classified as healthy within corresponding clusters ( $P < 0.0005$ ) (Table 1). Median pseudothecia density at North Fork was estimated to be 6.5 times higher than at Acey Creek (95% confidence interval from 4.1 to 10.2) and Juno Hill was 8.5 times higher than Upper Stone (3.0 to 24.5). Of the sites with mild to moderate levels of disease, the proportion of stomata occupied by pseudothecia at Coal Creek (moderate disease) was about twice that of Acey Creek (healthy, 1.1 to 3.4) in the North Cluster. However, the mildly diseased Cedar progeny site actually had a median pseudothecial density only about half that of the site rated as healthy in the South Cluster (0.37 to 0.92;  $P = 0.01$ ); both were also statistically equivalent to the moderately diseased Salal site.

**Ergosterol content.** Ergosterol reflected differences in disease severity between sites in the Tillamook and South clusters ( $P <$

0.05; Table 1). The median ergosterol content at Juno Hill was estimated to be four times greater than at Upper Stone (95% confidence interval from 2.8 to 5.6 times). In addition, the median ergosterol content at the mildly diseased Lower Stone was about 1.7 (1.2 to 2.4) times greater than the healthy site in that cluster. In the South Cluster, ergosterol content was similar for the mild disease (Cedar) and moderate disease (Salal) sites, but was significantly less in the healthy site (Limestone) (Table 1).

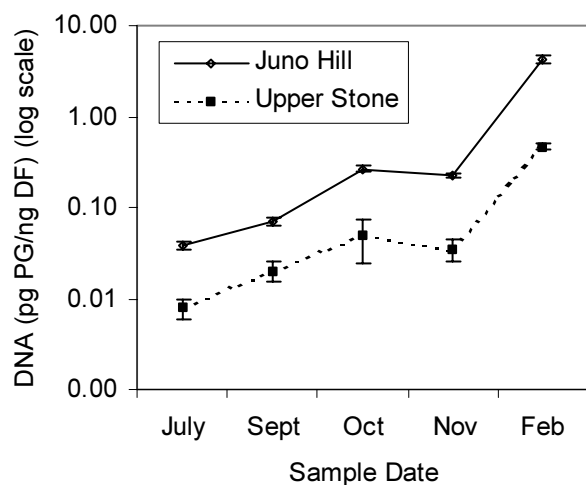
**Dot blot hybridization assay.** Of six candidate probes, a 900-base fragment generated from primer B02 was the most specific for *P. gaeumannii* while still retaining enough sensitivity to detect the fungus in lightly infected needles. When tested against purified DNA from a variety of fungi commonly isolated from or found on Douglas-fir, the 900-base probe hybridized strongly to *P. gaeumannii* (Fig. 1) but also hybridized weakly to the fungus *Rasutoria pseudotsugae*, a close relative of *P. gaeumannii* (27; L. M. Winton, J. K. Stone, and E. M. Hansen, unpublished data). The other needle fungi tested were not detected, and the probe did not bind to purified Douglas-fir DNA, which of course would be present in any foliage sample.

In each site cluster, there were significant differences in estimated *P. gaeumannii* DNA between the most and least severely diseased sites ( $P < 0.05$ ; Table 1). North Fork, Juno Hill, and Salal were estimated to be 2.4 (95% confidence interval: 1.4 to 4.3;  $P = 0.0002$ ), 4.3 (2.9 to 6.6;  $P < 0.0001$ ), and 2.2 (1.0 to 4.8;  $P = 0.024$ ) times higher, respectively, in *P. gaeumannii* DNA than the corresponding healthy sites within their clusters.

**TaqMan quantitative PCR.** Both sets of needles yielded similar results, were well correlated ( $R = 0.86$ ), and revealed significant differences between sites with low levels of disease compared with sites with moderate and severe disease within clusters ( $P < 0.05$ ; Table 1). For TAQMAN2, median *P. gaeumannii* DNA at North Fork, Juno Hill, and Salal were estimated to be approximately 4.5 (95% confidence interval: 3.1 to 6.4;  $P < 0.0001$ ), 9.7 (3.7 to 25.6;  $P < 0.0002$ ), and 1.7 (1.0 to 2.9;  $P = 0.026$ ) times higher, respectively, than at the respective healthy sites.

Differences in *P. gaeumannii* DNA among sites could be estimated well before pseudothecium formation (Fig. 2). For example, *P. gaeumannii* DNA was detected within 6 weeks of infection at Juno Hill and within 4 weeks at Lower Stone.

**Comparison of methods.** All four of the methods used to estimate average colonization were significantly correlated ( $P < 0.0001$ ; Table 2). Correlations were high, but somewhat variable, between pseudothecia density and quantitative PCR on the two sets of needles. Pseudothecia density had a higher correlation with DNA extracted from the same set of needles (TAQMAN2) than with a random sampling of needles (TAQMAN1). Ergosterol and the dot blot analysis, the two less specific assays, both had similar and lower correlations to pseudothecia density than did quantitative PCR. Dot blot analysis, which is more specific for *P. gaeumannii* than ergosterol, had a slightly higher correlation with



**Fig. 2.** TaqMan estimates of *Phaeocryptopus gaeumannii* (PG) DNA in current year Douglas-fir (DF) needles collected periodically from the least diseased and most severely disease sites in the Tillamook cluster. Vertical bars represent standard error of the means. Bud break and ascospore infection began in late May 1999 at the Juno Hill site, about 6 weeks before the first sample collection. Bud break at Upper Stone began about 4 weeks before the first collection date. Recognizable pseudothecial initials were first observed in February 2000 at Juno Hill and in April 2000 at Upper Stone.

**TABLE 2.** Pearson product moment correlation coefficients for comparisons between pseudothecia density, TaqMan quantitative polymerase chain reaction, ergosterol, and DNA probe methods for quantification of *Phaeocryptopus gaeumannii* colonization of Douglas-fir foliage<sup>x</sup>

	Pseudothecia	TAQMAN1 <sup>y</sup>	TAQMAN2 <sup>z</sup>	DNA probe
Pseudothecia	...	...	...	...
TAQMAN1	0.79	...	...	...
TAQMAN2	0.85	0.86	...	...
DNA probe	0.53	0.67	0.58	...
Ergosterol	0.47	0.56	0.42	0.65

<sup>x</sup> All correlations coefficients are significant at  $P < 0.0001$ . Means for 10 or 20 trees in each of nine sites were compared.

<sup>y</sup> TAQMAN1 was a random sampling of needles on which the dot blot hybridization probe assay was also performed.

<sup>z</sup> Pseudothecia density was determined for this same sample prior to DNA extraction.

pseudothecia density than did ergosterol. Dot blot analysis was better correlated with the quantitative PCR assay performed on the same DNA extraction (TAQMAN1) than it was with the second quantitative PCR experiment (TAQMAN2). Correlations between chlorosis and pseudothecia density, quantitative PCR, and dot blot analysis were significant ( $P < 0.0001$ ) and similar ( $0.46 \leq R \leq 0.49$ ). However, increased chlorosis appeared to be slightly better correlated with ergosterol content ( $R = 0.52$ ). A similar situation was seen with correlations between canopy density and ergosterol ( $R = -0.53$ ), while canopy density correlated less well with the three more specific methods ( $-0.36 \leq R \leq -0.48$ ).

## DISCUSSION

Improved methods to detect and quantify infection by *P. gaeumannii* will facilitate many studies investigating the effects of foliage infection and colonization by *P. gaeumannii*. This will aid in evaluating disease resistance in trees and enable investigation of the progress of needle colonization over time and in relation to various nutritional, environmental, and chemical factors.

Although fruiting body abundance (pseudothecia density) is well correlated with symptoms such as chlorosis and needle retention, it has severe limitations. No expensive materials other than a microscope are needed, but visual counting of stomata and pseudothecia is very time-consuming, tedious, and can be subject to misidentification errors. The common Douglas-fir needle fungus *Rasutoria pseudotsugae*, as well as species of *Rhizosphaera* and *Stomiopeltis* are also frequently found on Douglas-fir needles and can be mistaken for *P. gaeumannii* unless workers are well-trained and observant. In addition, because it is impossible to count every stomate on each needle in a sample, only a small fraction of the available stomata are examined. This has important statistical considerations when pseudothecia are unevenly dispersed over the needle surface, even if stomatal rows are randomly sampled. In this case, a randomly assigned stomatal row might easily give either erroneously high or low estimates, depending on the pattern of pseudothecial clustering. Another problem with counting pseudothecia is that early phases of infection, before pseudothecia develop, cannot be assessed.

Measurement of ergosterol is less cumbersome than counting pseudothecia on individual needles and is sensitive enough to quantify fungal biomass in needles not yet producing pseudothecia. One limitation of ergosterol, however, is that it is non-specific. Because it is a component of fungal cell membranes, any fungal species on or within needles will contribute to the total ergosterol and could lead to erroneous estimates of *P. gaeumannii* colonization. In trees and sites with moderate to heavy colonization by *P. gaeumannii*, the relative contribution of other fungi within and on needle surfaces is probably minimal, and ergosterol might provide a good approximation of *P. gaeumannii* biomass. A second problem with the use of ergosterol is variation in ergosterol content of cells over time in response to temperature, availability of nutrients, and age of cells. This effect has the potential to confound field experiments and also demands that samples be processed quickly. However, ergosterol may still be a useful technique to estimate fungal biomass in needles, because it is relatively inexpensive, rapid, and can be applied to a large number of samples. It would be most useful in combination with pseudothecia counts or quantitative PCR as a separate indication of total fungal biomass present.

Hybridization of labeled DNA probes to dot blotted sample DNA has the potential to be very specific. However, in this study, all nine of the probes tested also cross-reacted with fungi closely related to *P. gaeumannii*. The lack of specificity most likely was a consequence of the length of the probe necessary to generate a signal with enough sensitivity to detect DNA in low amounts. We tested probes ranging from approximately 300 to 900 bases in length, and all hybridized in varying degrees to at least one of the

other fungi tested. In an attempt to increase the specificity, we also tested a radiolabeled oligonucleotide that was homologous to an 18-base region in the internal transcribed spacer of nuclear ribosomal genes (data not shown). Although this probe was highly specific, it did not detect *P. gaeumannii* in any but the most heavily infected needles.

Real-time quantitative PCR is a relatively new technology that derives specificity from three oligonucleotides, two serving as PCR primers and one fluorescently labeled internal probe. Real-time quantitative PCR has the advantage of speed, technical simplicity, very low detection limits, and unparalleled specificity. The method capitalizes on the sensitivity of PCR and in some systems has been reported to have a lower detection limit of a single genome unit (one DNA molecule) (21,24). In addition to the low detection limit, TaqMan PCR has been shown to accurately quantify parasitic microorganisms in host tissue over a range of several orders of magnitude (6). In this study, our standards displayed a linear range of 5 orders of magnitude. This large range enables direct comparisons between samples with widely differing amounts of infection without complications of handling samples multiple times and the possibility of introducing error when diluting them to an appropriate concentration.

Quantitative PCR is the only method tested to date that can detect and quantify *P. gaeumannii* early in the disease cycle. Although sample collection did not begin early enough to capture the initiation of the infection cycle, significant differences in *P. gaeumannii* DNA between the severely diseased and healthy sites in the Tillamook cluster were detectable by July 1999 (Fig. 2). At this time, needles were less than 6 weeks old. Ascospore release and subsequent infection of trees at the nearby Salal site occurred between middle June and early July in 1999 (J. K. Stone, unpublished data). Differences in *P. gaeumannii* DNA are therefore detectable within only a few weeks of initial infection. Because the rate of increase was not different between the two Tillamook sites (Fig. 2), it is possible that the higher amount of *P. gaeumannii* DNA at Juno Hill is the result of greater initial inoculum amount rather than faster growth rate. All methods displayed relatively large differences in variance among sites, and may simply reflect heterogeneity within tree crowns and site conditions or may be a consequence of host genetic variability.

The quantitative methods presented here illustrate that it is possible to quickly detect the presence of *P. gaeumannii* and to assess the total amount of *P. gaeumannii* colonization of Douglas-fir needles at any time of year, regardless of the presence of pseudothecia. This should provide very sensitive standardized methods for comparing total *P. gaeumannii* DNA within infected foliage that can be used in a number of planned or already in progress studies. The real-time PCR assay shows particular promise for large studies because it is extremely cost effective. Considering material and labor expenses involved in this study, real-time PCR was almost half the cost of counting pseudothecia and a quarter the cost of ergosterol analysis. Furthermore, this technique could be readily adapted to the study of other plant pathogens in host tissue, particularly those with relatively long latent periods.

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